Table VI. Third-order Rate Constants for the Oxidation of Halides by Hypochlorite and by Hypohalous Acids

reactants	k, M^{-2} s^{-1}	ref
$H^+ + OCl^- + Br^-$	3.65×10^{10}	*a*
$H^+ + OCl^- + I^-$	4.4×10^{15}	*b*
$H^+ + HOCl + Cl^-$	2.8×10^4	*c*
$H^+ + HOCl + Br^-$	1.3×10^6	*a*
$H^+ + HOBr + Br^-$	1.6×10^{10}	*d*
$H^+ + HOI + I^-$	4.4×10^{12}	*d*

[a] This work. [b] Reference 2. [c] Reference 13. [d] Reference 5.

transfer reactions (i.e. why HO^+ and H_2O^{2+} should react much more rapidly than the oxygen atom).

Reactions are known in which a halide reacts as a nucleophile at an oxygen atom, for example the bromide reactions with hydrogen peroxide:[16]

$$H_2O_2 + Br^- \xrightarrow{2.3 \times 10^{-5}\ M^{-1}\ s^{-1}} OH^- + HOBr \quad (22)$$

$$H^+ + H_2O_2 + Br^- \xrightarrow{1.4 \times 10^{-2}\ M^{-2}\ s^{-1}} H_2O + HOBr \quad (23)$$

In these cases OH^- and H_2O are leaving groups, in a manner similar to structures I and II where OH^+ replaces Cl^+. On the other hand, the above H_2O_2 rate constants are factors of 1.5×10^8 and 1.1×10^8 slower than the corresponding reactions of HOCl. This is consistent with a less favorable nucleophilic attack by bromide ion at oxygen as compared to chlorine.

In the Cl^+-transfer mechanism, protonation of the oxygen atom greatly assists the rate because OH^- and H_2O are suitable leaving groups. Since the O^{2-} ion is not an appropriate leaving group, water must donate a proton if the k_0 path ($H_2O + OCl^- + Br^-$) also occurs by Cl^+ transfer.

Table VI summarizes the third-order H_3O^+-assisted rate constants for the reactions of hypochlorite and hypohalous acids with halide ions. The relative reactivity for $H^+ + OCl^-$ with halides is $I^- \gg Br^-$ and for $H^+ + HOCl$ with halides is $Br^- \gg Cl^-$. The increase in reactivity with $I^- \gg Br^- \gg Cl^-$ reflects the relative nucleophilicity of the halide ions. The rate constants for $H^+ + HOX + Br^-$ show that HOBr is more reactive than HOCl, even though the HOCl reaction is more favorable thermodynamically. The relative reactivity of HOCl, HOBr, and HOI parallels the ability to more easily expand the coordination of X and transfer X^+ to the halide ion.

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Registry No. Br^-, 24959-67-9; OCl^-, 14380-61-1; HOCl, 7790-92-3.

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Removal of Iron from Transferrin by Pyrophosphate and Tripodal Phosphonate Ligands

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The kinetics of iron removal from transferrin in 0.1 M hepes buffer at pH 7.4 and 25 °C have been studied by visible spectroscopy. Pseudo-first-order rate constants have been determined as a function of the ligand concentration for the ligands pyrophosphate, nitrilotris(methylenephosphonic acid), *N*-(phosphonomethyl)iminodiacetic acid, *N*,*N*-bis(phosphonomethyl)glycine, and nitrilotriacetic acid. The general equation that describes this ligand dependence is $k_{obsd} = k'[L]/(1 + k'[L]) + k''[L]$. The results are discussed in terms of two parallel pathways for iron removal, one that is first order in ligand and another that shows saturation kinetics. Iron removal by the phosphonic acids proceeds predominately through the saturation pathway, while iron removal by the structurally related ligand nitrilotriacetic acid proceeds exclusively through the first-order pathway. Iron removal by pyrophosphate is relatively rapid through both pathways, so that the saturation process predominates at low ligand concentration and the first-order pathway predominates at higher ligand concentrations.

Introduction

Serum transferrin is the primary mammalian iron transport protein. The protein has been extensively studied, and several recent reviews of transferrin chemistry are available.[1-3] Transferrin consists of two major lobes, designated N-terminal and C-terminal, with a single high-affinity iron-binding site associated with each lobe. These sites are well separated, and although they are very similar, they are not identical.

Serum transferrin belongs to a small class of proteins that includes ovotransferrin and lactoferrin. The distinguishing characteristic of these proteins is the requirement of a synergistic anion for effective metal binding. Under physiological conditions the anion is (bi)carbonate, which binds simultaneously to the iron and to cationic side groups on the protein to form an Fe–HCO_3–Tf ternary complex. Under carbonate-free conditions, reasonably stable ternary complexes can be formed with a variety of other anions.[4]

At sites of iron utilization, ferric transferrin binds to specific cell membrane receptors and releases its iron, although the process by which iron is removed from the very stable transferrin complex is still not clearly understood.[3] Iron removal from transferrin could also be important in chelation therapy for chronic iron overload. Certain genetic disorders such as β-thalassemia require frequent transfusions of whole blood. Since the body is unable to excrete the iron contained in this blood, it accumulates to lethal levels in organs such as the heart.[5] As a serum protein, transferrin is readily accessible to therapeutic chelating agents, but the rate of iron removal by desferrioxamine B, the current drug of choice, is quite slow.[5,6] Thus there is considerable interest in ligands that can remove iron from transferrin more quickly.

Rates of iron removal by several classes of ligands, including phosphonic acids,[7] catecholates,[8,9] hydroxamates,[10,11] and pyrophosphate,[12,13] have recently been reported. In all cases a hyperbolic dependence of the rate of iron removal on the concentration of ligand was reported. In enzyme kinetics this type of

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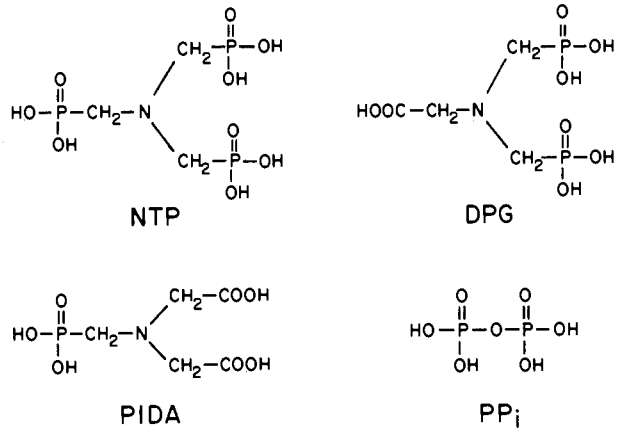
Chart I



saturation effect is typically ascribed to a preequilibrium between the enzyme and substrate, followed by a rate-limiting reaction of the enzyme–substrate complex to give product and regenerate the enzyme. A similar preequilibrium between ferric transferrin and the incoming ligand has been suggested,[8,11] and a ligand–Fe–HCO_3–Tf quarternary intermediate has been observed in studies on the formation of ferric transferrin from the reaction of the apoprotein with ferric complexes of pyrophosphate and acetohydroxamic acid.[10,12] However, spectroscopic evidence for formation of such an intermediate complex during iron removal has been observed only during the very slow reaction with the hydroxamate siderophore aerobactin.[11]

An alternative mechanism for iron removal has been proposed[10,12] that accounts for the lack of detectable concentrations of the ligand–Fe–HCO_3–Tf quarternary intermediate during iron removal by introducing a rate-limiting conformational change in the protein:

$$\text{Fe–Tf} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Fe–Tf*} \quad (1)$$

$$\text{Fe–Tf*} + \text{L} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Fe–Tf–L} \quad (2)$$

$$\text{Fe–Tf–L} \xrightarrow{k_3} \text{Fe–L} + \text{Tf} \quad (3)$$

Fe–Tf and Fe–Tf* refer to "closed" and "open" conformations of the ferric transferrin complex, and Tf refers to the binary HCO_3–transferrin species. Both this conformational change mechanism and a simple preequilibrium mechanism predict an equation for the apparent first-order rate constant having the general form

$$k_{obsd} = \frac{k'[\text{L}]}{1 + k''[\text{L}]} \quad (4)$$

where the definitions of k' and k'' differ for the two possible mechanisms.

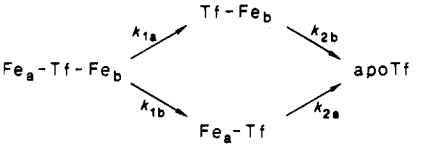
This paper reports continuing research on iron removal by phosphonic acids. Kinetic studies have been conducted using pyrophosphate, nitrilotriacetic acid (NTA), and the series of tripodal phosphonate analogues of NTA shown in the Chart I. The results indicate that eq 4 does not adequately describe the ligand dependence of iron removal and that a second term which is first order in ligand must be added.

Experimental Section

Reagents. Purified human serum transferrin was purchased from Calbiochem and further purified by using published procedures.[14] The tetrasodium salt of pyrophosphate was purchased and used without further purification.

Nitrilotris(methylenephosphonic acid) (NTP) was prepared by a Mannich reaction involving ammonium chloride, formaldehyde, and phosphorous acid by using published procedures.[15] The product was characterized by melting point and by ^1H and ^{31}P NMR spectroscopy. Anal. Calcd for $C_3H_{12}NO_9P_3$: C, 12.0; N, 4.68; H, 4.01; P, 31.1. Found: C, 12.0; N, 4.59; H, 3.93; P, 31.1.

Scheme I



***N*,*N*-Bis(phosphonomethyl)glycine (DPG).** DPG was purchased from Sigma as an impure crystalline material. The ligand was recrystallized by adding a 5-fold volume of acetone to a concentrated hot aqueous solution of DPG. The final product was characterized by a single line in the ^{31}P NMR spectrum. Anal. Calcd for $C_4H_{11}NO_8P_2$: C, 18.3; H, 4.14; N, 5.32; P, 23.6. Found: C, 17.9; H, 4.18; N, 5.65; P, 23.9.

***N*-(Phosphonomethyl)iminodiacetic Acid (PIDA).** PIDA was synthesized by reaction of iminodiacetic acid with excess (chloromethyl)-phosphonic acid according to published procedures.[16] The ligand was partially purified by precipitation as the lead(II) complex, followed by removal of the lead by bubbling H_2S through an aqueous suspension of the complex. However, even after repeated purification by precipitation of the lead salt, the product still contained ~13% of an impurity tentatively identified as (hydroxymethyl)phosphonic acid, which is formed during the reaction by hydrolysis of the starting chloro derivative. Anal. Calcd for $C_5H_{10}NPO_7 \cdot 0.3\ CH_5PO_4$: C, 24.42; H, 4.45; N, 5.37; P, 15.44. Found: C, 24.6; H, 4.50; N, 5.30; P, 15.8.

Procedures. Visible absorbance measurements were made with a Varian 2290 ultraviolet–visible spectrophotometer equipped with a jacketed sample holder connected to an external circulating water bath. All kinetic data were collected on samples at 25 °C in 0.100 M *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (hepes), which was adjusted to pH 7.4 with concentrated NaOH.

Results

The removal of ferric ion from transferrin was monitored by following the decrease in the iron–phenolate charge-transfer band of ferric transferrin at 465 nm. The ferric–phosphonate complexes have a very weak visible absorbance at 465 nm, such that the absorbance following complete removal of the iron from transferrin (A_∞) was approximately 10% of the original absorbance of diferric transferrin. Previous studies have been based on Scheme I for irreversible iron removal. Baldwin[17] has derived equations giving the concentration of each form of transferrin in terms of C_0, the concentration of diferric transferrin at $t = 0$, and the four rate constants from Scheme I.

$$[\text{Fe}_a\text{–Tf–Fe}_b] = C_0 e^{-(k_{1a}+k_{1b})t} \quad (5)$$

$$[\text{Fe}_a\text{–Tf}] = \frac{C_0 k_{1b}}{k_{1a} + k_{1b} - k_{2a}}(e^{-k_{2a}t} - e^{-(k_{1a}+k_{1b})t}) \quad (6)$$

$$[\text{Tf–Fe}_b] = \frac{C_0 k_{1a}}{k_{1a} + k_{1b} - k_{2b}}(e^{-k_{2b}t} - e^{-(k_{1a}+k_{1b})t}) \quad (7)$$

In previous studies the emphasis has been on evaluating possible cooperativity between the two sites.[9,17,18] However, to simplify the calculations it has been necessary to assume that $k_{2a} \cong k_{2b}$, so that iron removal can be described by two apparent rate constants, $m_1 = k_{1a} + k_{1b}$, and $m_2 = k_{2a} = k_{2b}$. Since these studies have failed to detect significant cooperativity, we have adopted a different treatment in which we assume noncooperativity, but make no assumption regarding the relative magnitude of the rate constants for each site.

The absorbance at any time t is simply the sum of the contributions from the three iron-containing species.

$$A_t - A_\infty = 2\epsilon[\text{Fe}_a\text{–Tf–Fe}_b] + \epsilon[\text{Fe}_a\text{–Tf}] + \epsilon[\text{Tf–Fe}_b] \quad (8)$$

ϵ is the molar absorptivity per iron ($\epsilon = 2500\ M^{-1}\ cm^{-1}$). The assumption of noncooperativity means that $k_{1a} = k_{2a}$ and $k_{1b} =$

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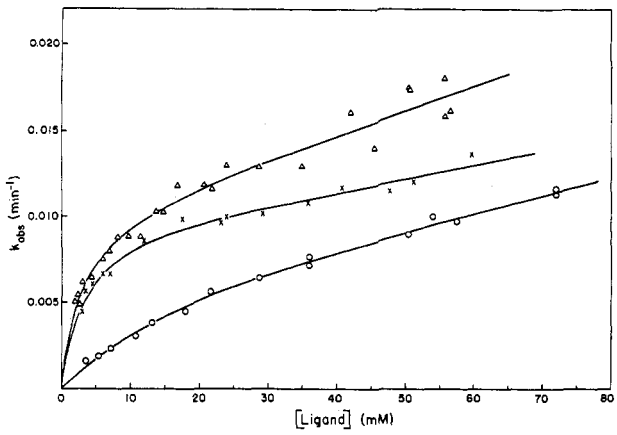

Figure 1. Plots of the pseudo-first-order rate constants for removal of ferric ion from serum transferrin in 0.10 M hepes, pH 7.4, 25 °C, as a function of ligand concentration for NTP (△), DPG (×), and PIDA (○). The lines are calculated fits based on eq 12 and the parameters listed in Table I.

k_{2b}; i.e., the rate of iron removal from a particular site is unaffected by iron binding at the other site. Thus eq 8 can be rewritten in terms of only two rate constants, k_a and k_b.

$$A_t - A_\infty = 2\epsilon C_0 e^{-(k_a+k_b)t} + \epsilon C_0(e^{-k_a t} - e^{-(k_a+k_b)t}) + \epsilon C_0(e^{-k_b t} - e^{-(k_a+k_b)t}) \quad (9)$$

The exponential terms in $(k_a + k_b)$ cancel, leaving a simple two-term exponential

$$A_t - A_\infty = \epsilon C_0(e^{-k_a t} + e^{-k_b t}) \quad (10)$$

Since ϵ is the molar absorptivity per site and C_0 is the molar concentration of diferric transferrin at $t = 0$, then $\epsilon C_0 = 0.5(A_0 - A_\infty)$, and eq 10 can be rewritten as

$$\frac{A_t - A_\infty}{A_0 - A_\infty} = 0.5e^{-k_a t} + 0.5e^{-k_b t} \quad (11)$$

The quantity $(A_t - A_\infty)/(A_0 - A_\infty)$ is a normalized reaction coordinate that is designated as R_t. The 0.5 coefficient for each exponential term reflects the fact that each rate constant describes removal of iron from a species that represents only half of the total iron pool. Apparent first-order rate constants were determined for a series of ligand concentrations from linear plots of ln R_t vs. time. Although eq 11 describes iron removal for all values of t, a simple semilog plot is linear only when the degree of iron saturation for the two sites is essentially the same. At the beginning of the reaction when the sites are equally occupied, $k_{obsd} = (k_a + k_b)/2$. Since the site specific rate constants k_a and k_b are usually different,[7,9,18] the degree of saturation for the two sites will begin to differ as the reaction proceeds. Thus the values of k_{obsd} were based on no more than the initial 30% of the reaction. At low ligand concentrations the plots are also affected by the approach to an equilibrium distribution of iron between the ligand and the protein, so that it was sometimes necessary to calculate k_{obsd} from data covering removal of less than 30% of total iron.

Plots of k_{obsd} vs. [ligand] are shown in Figure 1 for NTP, DPG, and PIDA. For NTP and DPG there is a rapid increase in k_{obsd} between 0 and 5 mM ligand, after which the plots begin to curve. Instead of reaching a flat plateau, as expected for classical saturation kinetics, the values of k_{obsd} continue to increase linearly with the increase in ligand. The initial increase in k_{obsd} for PIDA is much less than that for NTP or DPG. The departure from normal saturation kinetics is most clearly demonstrated by the PP_i data shown in Figure 2. The continuing increase in k_{obsd} at high ligand concentrations is inconsistent with eq 4. Thus it is proposed that the equation be modified by the addition of a first-order term to obtain eq 12.

$$k_{obsd} = \frac{k'[L]}{1 + k''[L]} + k'''[L] \quad (12)$$

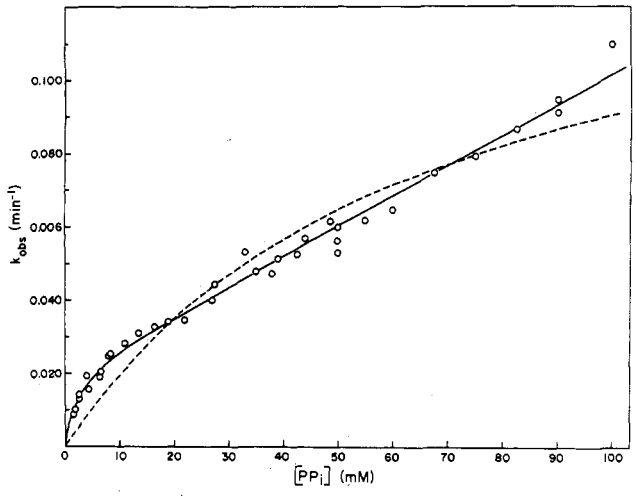

Figure 2. Plot of the pseudo-first-order rate constants for removal of ferric ion from serum transferrin in 0.10 M hepes, pH 7.4, 25 °C, as a function of the concentration of pyrophosphate. Open circles represent the actual data. The dashed line represents the calculated two-parameter fit based on eq 4. The solid line represents the calculated three-parameter fit based on eq 12 and the parameters listed for PP_i in Table I.

The data for all four ligands were fit to both eq 4 and 12 by using the general nonlinear least-squares program ORGLS.[19] The two- and three-parameter fits for PP_i, corresponding to eq 4 and 12, respectively, are shown in Figure 2. Equation 4 cannot account for both the initial rapid increase in k_{obsd} and the continuing increase at high PP_i concentrations and gives an R factor of 0.0500, where the R is defined as

$$R_n = \frac{\sum(k_{obsd} - k_{calcd})^2}{\sum(k_{obsd})^2} \quad (13)$$

where k_{obsd} and k_{calcd} are observed and calculated values of the apparent first-order rate constants for iron removal and n is the number of parameters used to fit the data. Equation 12 provides a much better fit of the experimental data, with an R factor of 0.0319. The curves in Figure 1 have been calculated by using eq 12.

The statistical validity of eq 12 was evaluated by using the R-factor ratio test.[20] For both the two- and three-parameter fits, an R factor was calculated as shown in eq 13. Increasing the number of adjustable parameters in a least-squares refinement should always lower the R factor. Thus a decrease in R does not necessarily indicate a more valid model. However, one can test the ratio of the R factors for two and three parameters to determine whether the improvement in R is statistically significant by using the test statistic $R = R_2/R_3$, where one expects R to be greater than 1.0. Significance can be tested by comparing R with the quantity

$$R_{b,n-p,\alpha} = \left[\frac{b}{n-p}F_{b,n-p,\alpha} + 1\right]^{1/2} \quad (14)$$

where n is the number of data points, p is the maximum number of adjustable parameters used to calculate R_p, b is the number of parameters held fixed to calculate R_{p-b}, and F is the element from the F distribution for b and $n - p$ degrees of freedom at the significance level α. If R exceeds $R_{b,n-p,\alpha}$, then one can reject the hypothesis that the two sets of parameters give equally valid fits of the data at the α significance level; i.e., one can conclude that the additional parameter is needed to adequately describe the data.

For PP_i, NTP, and DPG, the null hypothesis of equivalent fits for two and three parameters is rejected at $\alpha < 0.005$. The PIDA

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Table I. Rate Constants for Iron Removal from Serum Transferrin[a]

ligand	k', min^{-1} M^{-1}	k'', M^{-1}	k''', min^{-1} M^{-1}
PP_i	10.0 ± 3.1	490 ± 200	0.819 ± 0.030
NTP	3.98 ± 0.81	407 ± 120	0.137 ± 0.017
DPG	3.16 ± 0.045	337 ± 72	0.0667 ± 0.013
PIDA	0.333 ± 0.044	50 ± 24	0.087 ± 0.0143
LICAMS[b]	11.9	410	0
aerobactin[c]	0.35	120	0
acetohydroxamic acid[d]	1.6	32	0
NTA	0	0	0.158 ± 0.010

[a] Constants correspond to those in eq 12. [b] 1,5,10-Tris(5-sulfo-2,3-dihydroxybenzyl)-1,5,10-triazadecane; ref 8. [c] Reference 11. [d] Reference 10.

data are not as definitive, but the null hypothesis is still rejected at $\alpha = 0.05$. Thus it is clear that the additional term in eq 12 is necessary to adequately describe the dependence of k_{obsd} on the concentration of free ligand. The values of k', k'', and k''' obtained from the least-squares calculations are listed in Table I.

Kinetic data on the catecholate ligand 1,5,10-tris(5-sulfo-2,3-dihydroxybenzoyl)-1,5,10-triazadecane (LICAMS) and acetohydroxamic acid (AHA) were taken from ref 8 and 10, respectively, and analyzed by the procedures used for the phosphonates. The results should be interpreted cautiously, since there are only 5–6 data points for these ligands. In neither case can one reject the hypothesis that the two- and three-parameter fits are equivalent at $\alpha < 0.05$. Thus one can not assume that eq 12 is valid for all ligands.

The pseudo-first-order rate constants for iron removal by NTA were also determined for NTA concentrations ranging from 20 to 170 mM. In sharp contrast to the results with the phosphonic acids, the k_{obsd} values for the initial phase of iron removal show a simple first-order dependence on the concentration of free NTA. The values of k_{obsd} vs. [NTA] were analyzed by linear least squares to obtain the regression parameters of slope = 0.158 ± 0.010 min^{-1} M^{-1} and y intercept = −0.0003 ± 0.002 min^{-1}, with a Pearson correlation coefficient of 0.982. The slope corresponds to the second-order rate constant for iron removal from transferrin by NTA. The y intercept of essentially zero indicates that there is no parallel reaction pathway for iron removal that has been saturated at lower [NTA].

Discussion

The definitions for k', k'', and k''' from eq 6 in terms of fundamental rate steps obviously depend on the mechanism one chooses to describe the iron removal process. A simple preequilibrium mechanism requires the buildup of a quarternary L–Fe–HCO_3–Tf intermediate under saturating conditions. Results first with acetohydroxamic acid[10] and more recently with PP_i[12] appear to rule out such a mechanism for iron removal by these two ligands. Instead, Bates and co-workers have proposed the conformational change mechanism described by eq 1–3. By proposing that the rate-limiting step is a conformational change from an inert, "closed" form to a more labile, "open" form, one can account for the lack of a detectable concentration of the L–Fe–HCO_3–Tf quarternary intermediate during iron removal, even though this species is observed during iron donation to apotransferrin. When this conformational change is rate-limiting, then k_{max}, the maximum rate constant for the saturation process, will be equal to k_1 in eq 1, the forward rate constant for the transferrin conformational change. Since this rate constant is an intrinsic property of the protein, it should be independent of the ligand.

Table II lists k_{max} values for the ligands in this study as well as values calculated from literature data. Comparison of results from different studies is difficult because the effects of variables such as the salt concentration are not fully understood. It appears that the values for LICAMS, EDTP, HDEP, and PP_i tend to cluster around 0.025 min^{-1}. This is consistent with a common rate-limiting step. The rate constant for AHA is larger, which may be related to differences in experimental conditions. However, there was a substantial improvement in the fit of the AHA data

Table II. Values of k_{max} for the Saturation Pathway for Iron Removal from Transferrin

ligand	$10^3 k_{max}$, min^{-1}	half-life, min	medium
AHA[a]	51	14	20 mM hepes/20 mM HCO_3^-
EDTP[b]	30 ± 10	23	100 mM Tris
LICAMS[c]	29	24	100 mM Tris
PP_i	20 ± 8	35	100 mM hepes
HDEP[d]	19 ± 7	36	100 mM Tris
NTP	9.8 ± 2.9	71	100 mM hepes
DPG	9.4 ± 2.0	74	100 mM hepes
PIDA	6.7 ± 3.2	103	100 mM hepes
aerobactin[e]	2.9	238	10 mM hepes

[a] Acetohydroxamic acid.[10] [b] Ethylenediamine-*N*,*N*,*N*′,*N*′-tetrakis(methylenephosphonic acid); ref 7. [c] 1,5,10-Tris(5-sulfo-2,3-dihydroxybenzyl)-1,5,10-triazadecane; ref 8. [d] 1-Hydroxyethane-1,1-diphosphonic acid; ref 7. [e] Reference 11.

to eq 12 ($\alpha \simeq 0.1$), so the higher k_{max} for AHA may reflect a nonzero value for k'''.

T-Tests show that the k_{max} values for NTP, DPG, and PIDA are statistically equivalent. However, the larger k_{max} value for PP_i is significantly different from those for the three phosphonic acids. These data on the tripodal phosphonates and PP_i permit a direct comparison of k_{max} values from a single study where differences in experimental conditions are not a factor. This adds considerable confidence to the statistical analysis, which indicates that there is a significant difference between the k_{max} values for the tripodal ligands and the value for PP_i. Thus the rate-limiting step for iron removal by the phosphonic acids is slower than the rate of the protein conformational change. If one assumes simple saturation behavior and attempts to analyze the phosphonate data according to eq 4, then the disparity between k_{max} values for PP_i and the aminophosphonic acids becomes even greater. The conclusion that k_{max} is ligand-dependent holds regardless of which equation one chooses.

The value of k_{max} for PP_i determined from the least-squares fit of the data to eq 12 has a rather high uncertainty. This arises in part because the sum of squares of the residuals is dominated by the more extensive data in the linear range of the plot in Figure 2, such that the overall fit is less sensitive to individual values for k' and k''. The data from 18 to 100 mM PP_i have also been analyzed by linear least squares, where k_{max} corresponds to the y intercept of 0.018 ± 0.004 min^{-1}. The greater precision of this value adds more confidence to the significance of the differences between the k_{max} values for PP_i and the phosphonic acids.

It has been pointed out that the observation of reaction rates slower than the ferric transferrin conformational change does not contradict the basic conformational change mechanism.[7,12] It does require that the rate-limiting step for the slower iron removal by the phosphonic acids occur subsequent to the conformational change in the protein. If one assumes that the conformational change in ferric transferrin stays near equilibrium during the iron removal process, then one can derive that

$$k' = \frac{K_c k_2 k_3}{(K_c + 1)(k_{-2} + k_3)} \tag{15}$$

$$k'' = \frac{K_c k_2}{(K_c + 1)(k_{-2} + k_3)} \tag{16}$$

where the rate constants are defined by eq 2 and 3 and $k_1/k_{-1} = K_c$, the equilibrium constant for the conformational change.

The quantity $K_c/(K_c + 1)$ is equal to the fraction of ferric transferrin that exists in the "open" conformation and is designated as ϕ. The appearance of saturation kinetics in the phosphonic acid system with $k_{max} < k_1$ requires saturation at a step other than the conformational change. We assume this step is a preequilibrium formation of L–Fe–Tf. This requires that $k_{-2} \gg k_3$, so that one can substitute K_{eq} for k_2/k_{-2} and obtain

$$k' = \phi K_{eq} k_3 \tag{17}$$

$$k'' = \phi K_{eq} \tag{18}$$

It is interesting to compare the rate constants for the series of tripodal ligands NTP, DPG, PIDA, and NTA. Values of k_3, which corresponds to k_{max}, can be easily calculated as k'/k''. The k_3 values for all three phosphonates are statistically equivalent, so increasing the number of phosphonic acid moieties does not accelerate the rate of dissociation of the quarternary intermediate. This is consistent with the expectation that the rate of this step would be determined primarily by breaking of Fe–Tf bonds.[10]

Equation 18 shows that the value of k'' is proportional to the equilibrium constant for formation of the quarternary intermediate. The values in Table I show that, unlike k', this parameter does depend on the number of phosphonic acid groups. The k'' value for PIDA is significantly less than those for NTP and DPG. The lack of a saturation pathway for NTA can be understood as an extension of the trend of decreasing stability of the intermediate as one reduces the number of phosphonic acid groups.

The PIDA contained about 10% of an impurity that is believed to be (hydroxymethyl)phosphonic acid (HMP). Thus the comparison of PIDA values to those for NTP and DPG must be made with some caution. The iron-binding affinity for HMP is not expected to be large enough to result in direct iron removal by this ligand. The HMP could still alter iron removal rates by binding as an anion. However, our ongoing studies indicate that one would expect the major anion effect to be an increase in k'''. The actual value for k''' for PIDA is comparable to those of NTP and DPG. Thus we believe that the decrease in k'' for PIDA relative to the values for NTP and DPG is significant.

On the basis of preliminary spectroscopic data on carbonate-free ternary phosphonate–Fe–Tf complexes, we can rule out formation of the intermediate in eq 2 by ligand substitution for the synergistic bicarbonate anion. Two other types of intermediates are under consideration. One involves coordination of a single phosphonate group at the aquo/hydroxo site on the iron. If only one phosphonate group is involved, the change in k'' among the ligands would be primarily a statistical effect related to the number of phosphonic acid groups per molecule. There would be no advantage from a chelate effect for diphosphonates. The lack of a saturation pathway for NTA would reflect the weaker iron-binding affinity of a carboxylate group relative to that of a phosphonic acid. An alternative description of the quarternary intermediate involves binding of the highly negative phosphonate ligand as an anion to the protein, rather than by direct coordination to the iron. Chasteen has recently reported that there is a high concentration of cationic protein side groups near the iron-binding sites and that the interactions of these groups with perchlorate affect iron release kinetics.[21]

One should note that for ligands operating in the regime where the protein conformation change is rate limiting, the definition of k'' is different from that given in eq 18. Thus it is difficult to compare k'' for the tripodal phosphonates with the values for PP_i, EDTP, or HDEP. The value of k'' for PP_i also has a relatively high uncertainty, which hampers meaningful comparisons with the values for the phosphonic acids.

Rates of iron removal have been previously evaluated for the phosphonic acids NTP, ethylenediamine-*N*,*N*,*N*′,*N*′-tetrakis-(methylenephosphonic acid) (EDTP), and HDEP[7] and PP_i.[12,13] Slightly faster rates of iron removal by the phosphonic acids were observed. This difference may be due in part to the use of 0.1 M Tris–HCl as a buffer, rather than 0.1 M hepes, since chloride is known to affect the kinetics of iron removal.[17,22] However, the most important difference between this report and the previous paper on the phosphonates is the addition of a first-order term for iron removal to generate eq 12. This change is due primarily to an improvement in the method of data analysis, rather than a drastic change in the data themselves. In the previous study,[7] values of k' and k'' were determined graphically from a double-reciprocal plot of eq 4. This has the effect of heavily weighting points at low ligand concentration and bunching the data at high ligand concentrations near the origin. The result is that a gradual increase in k_{obsd} at higher ligand concentrations has little effect on the linearity of the double-reciprocal plot. In least-squares fits of the data to eq 4 and 12, more weight is given to the data at high ligand concentration, and the significant difference between the two- and three-parameter fits is readily apparent. We have also included 2–3 times the number of data points in this study, which helps to delineate more accurately the ligand dependence of k_{obsd}.

The phosphonate data from ref 7 have been reanalyzed by the least-squares methods used in this study. Equation 12 provides a significantly better fit for both EDTP and HDEP at a significance level of $\alpha = 0.05$. The improvement in the fit of the old NTP data is not significant at $\alpha = 0.1$, but this is primarily due to scatter in the data and the fewer number of data points. Thus we feel that eq 12 is generally applicable to iron removal by phosphonic acids.

It is well known that PP_i is exceptionally effective for facilitating the transfer of ferric ion from transferrin to other iron chelating agents such as desferrioxamine B.[5,13,23] In addition, it has recently been shown to remove iron effectively from transferrin in the absence of other chelating agents.[12] It now appears that this enhanced effectiveness is due primarily to the exceptionally high value of k''' in eq 12. In terms of the saturation process alone, PP_i is comparable to AHA and LICAMS, as it must be if a protein conformational change is the common rate-limiting step for all three ligands. It is also interesting to note that NTA has a very high value of k'''. The impression of slow iron removal by NTA is due to the lack of the rapidly saturating process associated with phosphonates, catecholates, and hydroxamates.

Although three studies have now reported a roughly hyperbolic dependence of K_{obsd} on $[PP_i]$, the rate constants vary considerably. The major portion of this discrepancy is due to differences in the methods of calculation. If the data reported here are treated as standard saturation kinetics by using eq 4 instead of eq 12, the value of k_{max} increases from 0.020 to 0.152 min^{-1}. An additional factor may be the concentration range over which data are collected, since the value of k_{obsd} continues to increase with increasing PP_i. A value of approximately 0.11 min^{-1} has been reported on the basis of data from 0 to 60 mM PP_i[13], compared to a value of 0.216 min^{-1} reported for data from 0 to 100 mM PP_i[12].

The physical significance of the first-order term in eq 12 is still in doubt. We have considered three possible explanations: (1) phosphonic acids react at each site by two parallel pathways, one which saturates at approximately 10 mM ligand and another which is first-order in ligand; (2) iron removal by the phosphonates follows saturation kinetics at one site, but is first-order in ligand at the remaining site; and (3) there is a single pathway that saturates at approximately 10 mM ligand, but allosteric binding of anionic ligands increases the value of k_3 as the ligand concentration increases.

The third possibility is suggested by the report of Folajtar et al.[24] that several anions, including PP_i, bind at nonsynergistic ("modifier") binding sites and change the ferric transferrin EPR spectrum. However, the equilibrium constant for PP_i binding to the modifier sites is known. If this binding were linked to the value of k_{obsd}, then one would expect a complete shift to the more labile form over the range 10–100 mM PYP. The linearity of the plot of k_{obsd} vs. $[PP_i]$ observed over this concentration range argues against a strong effect on the rate of iron removal from anion binding at the modifier sites. We cannot rule out allosteric effects from binding at other sites that remain far from saturation over the range 10–100 mM PP_i.

The existence of an independent first-order pathway for at least one site is indicated by a comparison of the data on NTA and PP_i. Without showing any signs of saturation, NTA is able to remove ferric ion from transferrin at a rate that exceeds the k_{max} for PP_i, even though this k_{max} presumably corresponds to the

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forward rate constant for the transferrin conformational change. Thus iron removal by NTA must proceed through a pathway that is not limited by this conformational change. Given the similarities between NTA and the tripodal phosphonic acids, it seems reasonable that this pathway would also be accessible to the phosphonic acids.

Since the k_{obsd} values are the average of k_a and k_b, one cannot resolve site-specific constants from the initial rates of iron removal from diferric transferrin. Thus we can not distinguish between models 1 and 2 described above. It is possible that the two pathways are associated with different sites. One site could react by a saturation pathway, while the other reacts by a simple first-order pathway. Alternatively, each site may react by parallel pathways. Studies on monoferric transferrins, which will distinguish between these two models, are in progress. Although the results are still preliminary, it appears that each site can react by both first-order and saturation pathways. We expect to be able to report more fully on site-specific behavior in the near future.

Ligands with bulkier coordinating groups such as catecholates or neutral groups such as hydroxamic acids do not appear to remove significant amounts of iron via the first-order pathway. This observation suggests that anion substitution to form a short-lived intermediate may be involved in the first-order pathway. Aisen et al. have proposed such an intermediate for the donation of iron from NTA to apotransferrin,[25] although Bates and Wernicke have suggested that the intermediate is a quarternary NTA–Fe–HCO_3–Tf complex.[26] Direct anion substitution has also been proposed to explain the observations that NTA and PP_i accelerate both the transfer of iron from transferrin to other chelating agents[27] and the exchange of bicarbonate in the Fe–HCO_3–Tf ternary complex.[28] In contrast to a previous report,[29] our preliminary data indicate that PP_i, as well as the three tripodal phosphonic acids, will form a ternary Fe–L–Tf complex under CO_2-free conditions. Thus anion substitution should be considered as a pathway for iron removal by PP_i and the phosphonic acids.

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